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Identification of novel reaction products of methylene-bis-phenylisocyanate ("MDI") with oxidized glutathione in aqueous solution and also during incubation of MDI with a murine hepatic S9 fraction

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Abstract

Methylene diphenyl diisocyanate (MDI) is an important industrial chemical and asthmagenic respiratory sensitizer, however its metabolism remains unclear. In this study we used LC-MS and LC-MS/MS to identify novel reaction products of MDI with oxidized glutathione (GSSG), including an 837 *m/z* $[M+H]^+$ ion corresponding to GSSG bound (via one of its N-termini) to partially hydrolyzed MDI, and an 863 *m/z* $[M+H]^+$ ion corresponding to GSSG cross-linked by MDI (via its two γ-glutamate N-termini). Further studies with heavy isotope labeled and native reduced glutathione (GSH) identified an $[M+H]^+$ ion corresponding to previously described mono(GSH)-MDI, and evidence for "oligomeric" GSH-MDI conjugates. This study also investigated transformational changes in MDI after incubation with an S9 fraction prepared from murine liver. LC-MS analyses of the S9 reaction products revealed the formation of $[M+H]^+$ ions with *m/z*'s and retention times identical to the newly described GSSG-MDI (837 and 863) conjugates and the previously described mono(GSH)-MDI conjugates. Together the data identify novel biological transformations of MDI, which could have implications for exposure-related health effects, and may help target future in vivo studies of metabolism.

Graphical Abstract

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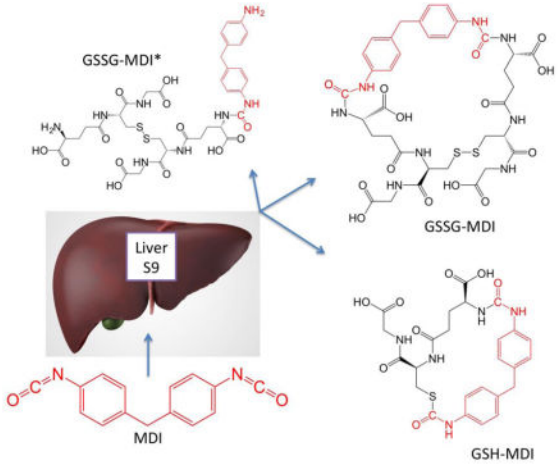

INTRODUCTION

MDI, a widely used chemical essential to numerous industries, is recognized to cause occupational asthma (1, 2). Workplace exposure levels are the best recognized risk-factor for disease development, yet methods for exposure surveillance remain limited (3, 4). A better understanding of MDI metabolism may identify biologically transformed chemical structures that could provide the basis of exposure biomonitoing efforts towards disease prevention, and might play a role in pathogenic responses.

Glutathione, a major airway antioxidant critical to metabolism of numerous endogenous and xenobiotic compounds, has been hypothesized to play a role in MDI metabolism (5). In vitro, MDI reacts rapidly with the reduced form of glutathione (GSH), resulting in quasi-stable, reversible thiocarbamate linkages between MDI's isocyanate groups and the free thiol of reduced GSH (6–10). The major GSH-MDI reaction products have been identified as *S*-linked bis(GSH)-MDI, mono(GSH)-MDI* (where * represents partially hydrolyzed MDI), and mono(GSH)-MDI, which may be stabilized by cyclization (6, 9). The *S*-linked GSH-MDI reaction products possess the capacity to transcarbamoylate self-proteins, resulting in antigenic changes recognized by the host's immune system (9). In vivo, respiratory tract exposure to GSH-MDI induces asthmatic pathology in sensitized hosts (8). Given the relatively high concentration of GSH in the lower airway fluid, it may provide a portal of chemical entry into the body, as well as a pathway for the development of immune activation (11).

In contrast to reduced GSH, the reactivity of MDI with oxidized glutathione (GSSG) remains undefined. Cross linkage of GSSG's γ-glutamate groups, or their conjugation with partially hydrolyzed MDI, should result in stable (*N*-linked) GSSG-MDI reaction products, lacking the pathogenic transcarbamylating potential of *S*-linked GSH-MDI. Thus, while GSH-MDI reaction products may retain pathogenicity, GSSG reactivity with MDI may result in chemical neutralization.

While GSH conjugation and metabolism of mono-isocyanates via the mercapturic acid pathway has been established as a major route by which these chemicals are excreted in vivo, the role of glutathione in metabolism of MDI (and other diisocyanates) remains unclear (12–15). Many studies to date have inferred diisocyanate excretion in urine based on the levels of the corresponding diamine released following acid or base hydrolysis (16–18). However, few studies have investigated the form of chemical excreted or how it gets transported from the site of exposure into the urine. A single study by Gledhill et al, in which rats were exposed to aerosolized ^{14}C-MDI, identified hydrolyzed, acetylated and oxidized metabolites of MDI in urine and feces (19). However, oral absorption (due to grooming during the post-exposure period) represented the major route of chemical entry into the animals in this study, despite the intended delivery to the lower respiratory tract (where GSH levels are relatively higher). Furthermore, sample workup including solid-phase extraction under low and high pH conditions (pH 2/10) and reluxing could have influenced the detection of pH and temperature-sensitive GSH-MDI conjugates (6, 9). In a single study by Pauluhn et al, when rats were intratracheally exposed to bis(GSH)-MDI adducts, the diamine corresponding to MDI was measurable in the urine in an exposure-dose dependent manner (20). In conjunction with related studies, these authors proposed a conceptual pathway by which diisocyanate chemical entry into the host lungs depends upon GSH adducts, rather than amines resulting from hydrolysis.

Given the complexity of in vivo studies and recognition of the technical problems encountered in prior studies of diisocyanate metabolism in small animal models, we undertook studies to begin investigating the metabolism of MDI in vitro. Based on the published data described above, the present study focused on potential glutathione-diisocyanate conjugates and possible hydrolyzed/acetylated metabolites that might form following MDI incubation with an hepatic S9 fraction (21), a commonly used in vitro system for assessing chemical metabolism. The findings are discussed with regard to the potential metabolism of MDI in vivo, exposure biomonitoring and disease pathogenesis.

METHODS

MDI reactivity with glutathione

MDI was reacted with oxidized, reduced and heavy-isotope labeled glutathione under conditions previously described (9). Briefly, 50 μL of 10% (w/v) MDI in acetone from JT Baker (Phillipsburg, NJ) was added to 25 ml of 10 mM glutathione solutions prepared in 200 mM sodium phosphate, pH 7.4 (final acetone concentration 0.2% v/v). The reactions were rotated end-over-end for 2 h at 37°C, centrifuged at 10 000 × *g*, and 0.2 μm filtered before mixing 1:1 with water containing 0.1% formic acid for analysis by LC-MS and LC-MS/MS. Reduced glutathione (CAS # 70-18-8), oxidized glutathione (CAS Number 27025-41-8) and 4,4′-methylenebis(phenyl isocyanate) (CAS # 101-68-8) were from Sigma-Aldrich (St. Louis, MO) and were of ≥98.0% purity. Heavy isotope labeled reduced glutathione, from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA), had a peptide purity of 85–90% (glycine $^{13}C_2$, >98%; ^{15}N, 96–99%).

Hepatic S9 studies

The 9,000 × *g* supernatants (S9) of liver homogenates from N=4 eight-week old male C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) was prepared according to the method of Wu and McKown (21). All animal usage was in conformance with a protocol approved by the Yale University School of Medicine's Institutional Animal Care & Use Committee. Briefly, the liver was minced and homogenized (twice at 10,000 rpm for 30 sec) in 4X the wet liver weight (4 mL/g) of 0.1M $NaPO_4$ (pH 7) using a Brinkmann Polyron. The homogenate was spun at 9,000 *g* for 30 min at 4°C, and the supernatant used immediately for testing. Metabolism was initiated by adding 10 uL of 10 % (w/v) freshly prepared MDI, or an equivalent amount of chemical that had been hydrolyzed in solution for 2hr at 37°C, to a final volume of 5 ml of S9 reaction mixture +/− cofactors required for NADPH regeneration (5 mM $MgCl_2$ 5 mM glucose-6-phosphate, and 0.5 mM NADP). Additional control experiments were carried out in the absence of an S9 fraction or chemical additives (solvent control). Following incubation at 37°C for 2 hrs, samples were filtered through a 3 kDa molecular weight spin column, mixed 1:1 with water containing 0.1% formic acid, and analyzed by LC-MS.

Liquid chromatography coupled mass spectrometry

LC-MS and LC-MS/MS were performed on an Agilent 6550 Q-TOF system coupled to an Agilent 1290 Infinity LC system, using a rapid resolution HT Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm), also from Agilent Technologies (Santa Clara, CA). Samples were mixed 1:1 in buffer A (water containing 0.1% formic acid) before 3 μL was loading and eluted over a 10 minute period, starting at time 0 with 5% buffer B (acetonitrile containing 0.1% formic acid), increasing to 40% buffer B between 0–5 min, 90% buffer B between 5–7 min, 95% buffer B between 7–9 minutes and back to 2% buffer B from 9–10 min. Positive electrospray ionization (ESI) was performed using the following parameters: gas temp- 280°C, gas flow- 11 l/min, nebulizer-40 psig, sheath gas temp- 350°C, sheath gas flow-11, Vcap-4000 V, nozzle voltage-2000 V, fragmentor voltage– 175 V, skimmer voltage 65 V, octopole RF peak voltage 750 V. For MS/MS analyses, the collision energy was automatically set using Agilent MassHunter Acquisition software according to the formula, slope × (*m/z*)/100 + offset; with the slope of 5 and offset of 2.5. The *m/z* values of all ions present in the mass spectra were corrected against two reference ions (purine, $[M+H]^+$ *m/z* 112.9856 and 1H, 1H, 3H tetra(fluoropropoxy)phosphazine, $[M+H]^+$ *m/z* 922.0097). The data acquisition range, from 110–1700 *m/z*, was acquired and analyzed using Mass Hunter Workstation software from Agilent. Quantitation of selected $[M+H]^+$ ions was calculated from extracted ion chromatograms (EICs), based on the relative area under the curve of peaks with defined *m/z* ratios and retention times. The significance of differences between studies conducted in the presence vs. absence of an NADPH regenerating system was determined by *t*-test.

RESULTS

Reactivity of MDI with oxidized glutathione (GSSG)

Initially, we studied the reactivity of MDI with purified GSSG in aqueous solution. When analyzed by LC-MS, GSSG-MDI reaction products were readily identified as new peaks

among the base peak chromatogram (BPC), as shown in Fig. 1 and supplemental materials available on line. The major GSSG-MDI reaction products include $[M+H]^+$ ions whose *m/z* ratios (837.25, 863.23, and 1475.39) and proposed structures are consistent with MS/MS fragmentation patterns observed upon collision induced dissociation (CID) (Figure 2 and supplemental materials). The basis for elution of a small portion of the 863.23 *m/z* ion with a slightly abbreviated retention time remains unclear. The $[M+H]^+$ ion with the 225.10 *m/z* is consistent with that expected for partially hydrolyzed MDI, however, the structure of the molecule and "survival" of one N=C=O group in aqueous solution at 37°C also remains unclear.

Reactivity of MDI with reduced glutathione (GSH) labeled with heavy isotope

To further investigate the reactivity of MDI with reduced forms of glutathione, we performed studies using heavy isotope labeled GSH (GSH^i). The mass difference (+3.0038 Da) between GSH^i and "native" GSH, together with prior data on GSH-MDI reaction products, was used to help characterize the range of GSH-MDI reaction products and model their molecular structures. As shown in Fig. 3, MDI-GSH^i reaction products were readily identified as new peaks among the LC-MS BPC, predominately as $[M+H]^+$ ions whose retention times and *m/z* ratios correspond to the previously described bis(GSH)-MDI, mono(GSH)-MDI, and mono(GSH)-MDI*, where * represents partially hydrolyzed MDI) (6, 9). In addition, we observed another $[M+H]^+$ ion whose *m/z* ratio (1431.42) was consistent with "oligomeric" GSH^i-MDI, (e.g. a molecule containing three GSH^i "cross-linked" by two MDI). Further LC-MS/MS with native GSH-MDI reaction products was performed to characterize this potential GSH-MDI oligomer. The data (see supplemental materials Fig. S4) provide evidence for candidate structures modeled in Fig 4 A & B, based on the 607.16 *m/z* $[M+H]^+$ daughter ion observed upon CID, which is consistent with that previously reported for bis(cys-gly)-MDI (5). However, the presence of additional oligomers with structures depicted in Fig. 4C & D could not be ruled out. Intramolecular rearrangement or in-source fragmentation of "oligomeric" GSH-MDI could result in the formation or release of mono(GSH)-MDI and may account for its elution at different time points.

Formation of GSSG-MDI*, GSSG-MDI, and mono(GSH)-MDI upon incubation of MDI with a liver S9 fraction

Preliminary LC-MS studies of MDI following incubation with an S9 fraction (data not shown) prompted us to focus on the potential biotransformation of MDI via reactivity with glutathione. Further LC-MS was performed on samples of MDI incubated with a murine liver S9 fraction and extracted ion chromatograms were generated for $[M+H]^+$ ions with *m/z*'s corresponding to the GSSG-MDI and GSH-MDI reaction products described above. As shown in Fig. 5, $[M+H]^+$ ions with *m/z*'s corresponding to GSSG-MDI*, GSSG-MDI and mono(GSH)-MDI are observed following incubation of MDI (but not control hydrolyzed MDI) with a liver S9 fraction, and exhibit identical retention times compared with standards generated using purified GSSG or GSH^i. The levels of the different GSH and GSSG-MDI products were not significantly affected when studies were performed in the absence of co-factors required for NADPH regeneration (see supplemental materials Fig S5).

DISCUSSION

This in vitro study of the occupational allergen, MDI, extends our understanding of the chemical's reactivity with glutathione, an important anti-oxidant of the lower airways involved in the metabolism of many endogenous compounds and xenobiotics. LC-MS/MS data reveal the potential for MDI to react with oxidized, as well as reduced forms of glutathione, resulting in several previously undescribed glutathione-MDI reaction products, including MDI cross-linked GSSG (863 *m/z*) and partially hydrolyzed MDI-GSSG (837 *m/z*). Further LC-MS studies support the formation of these novel GSSG-MDI conjugates, as well as the previously described mono(GSH)-MDI (9) following incubation of MDI with murine liver S9. The liver contains high concentrations of both reduced and oxidized non-protein bound glutathione and is also the central organ responsible for inter-organ GSH homeostasis, with sinusoidal GSH efflux as the major determinant of plasma and lung GSH (22, 23). Together, the data support growing evidence for glutathione as an important reaction target for MDI, which could play a role in its metabolism.

Although MDI reactivity with reduced glutathione has been previously described, MDI's capacity to react with oxidized glutathione has not been appreciated and involves important differences potentially relevant to MDI exposure-induced pathogenesis. GSH-MDI conjugates, formed by linkage of the GSH sulfhydryl group to one of MDI's isocyanate groups, are "reversible" and can transcarbamylate self-molecules, resulting in antigenic changes. In contrast, GSSG-MDI reaction products are formed by stable covalent linkage of the N-terminal amino acid of GSH to MDI's isocyanate groups and are not "reversible". Thus, the present data suggest the novel hypothesis that GSSG (but not GSH) might participate in MDI detoxification and that stable GSSG-MDI conjugates may serve as biomarkers of exposure, amenable to disease prevention efforts.

The present study also yields new insight into the range of possible GSH-MDI reaction products, including evidence for "oligomeric" GSH-MDI reaction products. While the exact chemical structure of such GSH-MDI oligomers remains uncertain, MS/MS analysis suggests at least a portion contain potentially reversible thiocarbamate linkages, and could undergo intra- or inter-molecular rearrangements. Another important observation of this study is the elution of mono(GSH)-MDI at three different time points under reverse phase LC-MS. It remains unclear if this elution pattern reflects different conformations of mono(GSH)-MDI, results from intra-molecular rearrangement of oligomeric GSH-MDI, or in-source fragmentation. Further studies, such as NMR analysis of mono(GSH)-MDI eluting at different time points, should help explain the observed elution profile, but are beyond the scope of the present study.

The strengths and weaknesses of the present study are important to recognize when interpreting the significance of the findings. The novelty of the study is one strength; while S9-mediated transformation of mono-isocyanates (e.g. decomposition products of fotemustine, carmustine, and other anti-cancer drugs) has been shown to involve GSH (12, 13), to the best of our knowledge, similar studies with diisocyanates have yet to be reported. Additional strengths of the present investigation include the use of LC-MS and LC-MS/MS to characterize MDI reaction products with glutathione under well controlled conditions in

vitro. Studies with purified GSSG, GSH, and heavy isotope labeled GSH provided guidance crucial to identifying glutathione-MDI reaction products following chemical incubation with a liver S9 fraction.

Weaknesses of the present investigation include the lack of experiments with co-factors required for some phase II metabolites (e.g. glucouronidated or sulfated), orthogonal approaches (e.g. NMR) to confirm the chemical structures proposed based on LC-MS/MS, and mass balance analyses, given MDI's likely reactivity with the abundant proteins in an S9 fraction (24, 25). Our studies did not detect the acetylated and hydroxylated MDI metabolites characterized by Gledhill et al (19), which could be due to technical issues discussed in the introduction. However, the data are consistent with studies by Pauluhn et al suggesting that GSH represents a portal of entry for diisocyanates (20). While a murine hepatic S9 fraction was used in the present study, MDI reactivity with GSH and GSSG occurs independent of enzymes (which may differ between species), and a human hepatic S9 fraction would also contain high levels of GSH and GSSG (26). Further studies will be needed to address the questions inspired by the present findings and their relevance to MDI metabolism in vivo in humans.

In summary, the important industrial chemical and asthmagenic respiratory sensitizer, MDI, was shown to react with glutathione in its oxidized, as well as its reduced state. Evidence for the generation of specific GSSG-MDI and GSH-MDI reaction products, following incubation of MDI with liver S9 fractions was supported by LC-MS, and LC-MS/MS data. The newly described reaction products of MDI with oxidized glutathione may represent stable (*N*-linked) metabolites, which could serve as exposure biomarkers and play a role in chemical detoxification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

BPC	base peak chromatogram
CID	collision induced dissociation
ESI	electrospray ionization
GSH	glutathione in its reduced state
GSSG	glutathione in its oxidized state
LC	liquid chromatography

MDI	methylene diphenyl diisocyanate
MS	mass spectrometry
S9	supernatants following centrifugation at 9,000 *g*
*****	partially hydrolyzed MDI

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Highlights

- Methylene diphenyl-diisocyanate (MDI) reacts with oxidized and reduced glutathione
- Oxidized glutathione (GSSG) forms stable *N*-linked conjugates with MDI
- Reduced glutathione (GSH) forms stable *N*- and reversible *S*- linkages with MDI
- Data suggest MDI conjugates to GSSG and GSH upon incubation with an S9 fraction
- MDI-GSSG conjugates may be stable metabolites, amenable to exposure surveillance

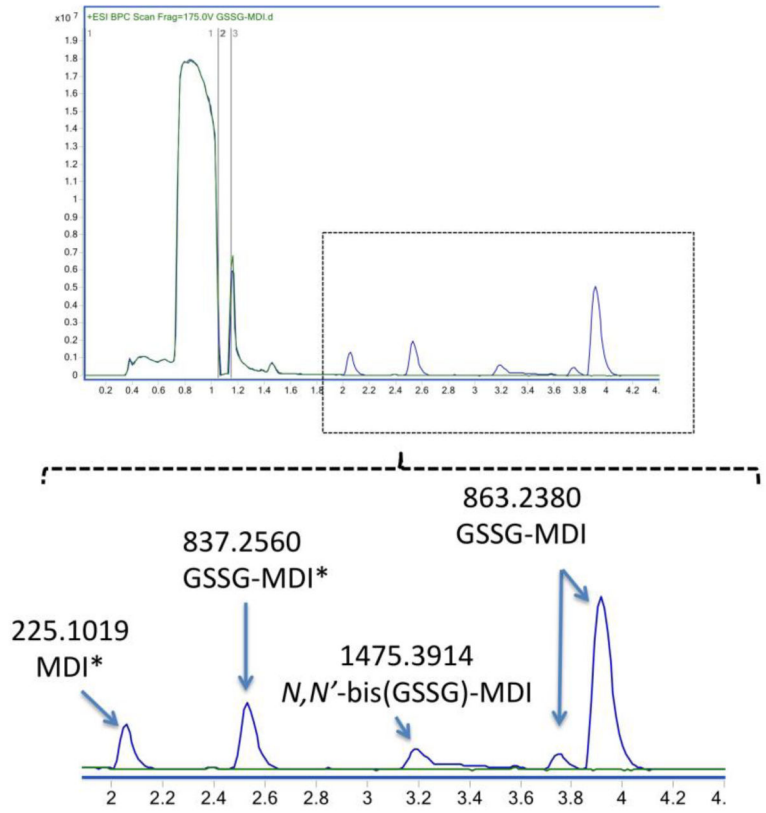

Figure 1.

MDI reactivity with oxidized glutathione (GSSG). MDI (blue line) or solvent control (green line) was reacted with purified GSSG and analyzed by LC-MS. The region of the base peak chromatogram (BPC) containing new ions with *m/z* values corresponding to different GSSG-MDI reaction products is highlighted, with corresponding ESI-MS readings overlaid. Relative ion signal intensity is depicted on the Y-axis and retention time in minutes on the X-axis. Data shown are representative of three independent experiments.

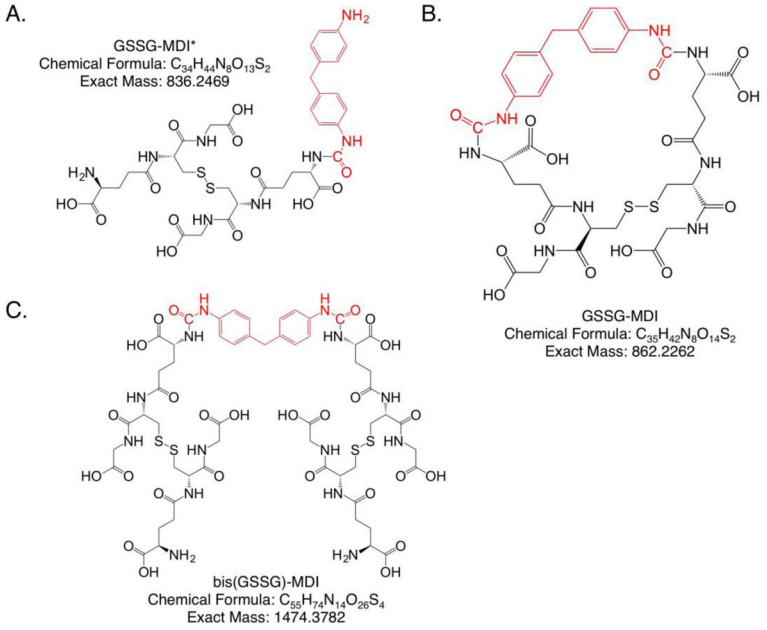

Figure 2.

Proposed structures for GSSG-MDI reaction products. Chemical structures for novel reaction products between MDI and GSSG are modeled based their exact mass and the fragmentation patterns observed upon collision induced dissociation during LC-MS/MS analyses (see supplemental materials for more information).

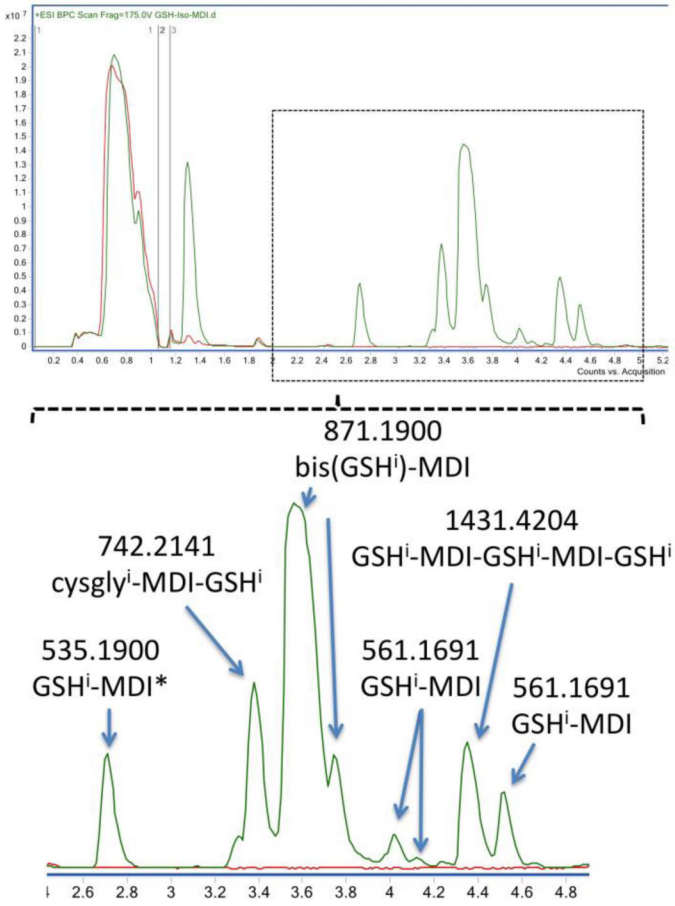

Figure 3.

MDI reactivity with reduced glutathione (GSH) labeled with heavy isotope. MDI (green line) or solvent control (red line) was reacted with heavy isotope labeled GSH (GSH^i) and analyzed by LC-MS. The region of the BPC containing new ions with *m/z* values corresponding to different GSH^i-MDI reaction products is highlighted, with ESI-MS readings overlaid. Ion signal intensity is depicted on the Y-axis and retention time in minutes on the X-axis. *The 742 *m/z* ion likely forms from cys-gly present at low levels in the starting material (not shown). Data shown are representative of three independent experiments.

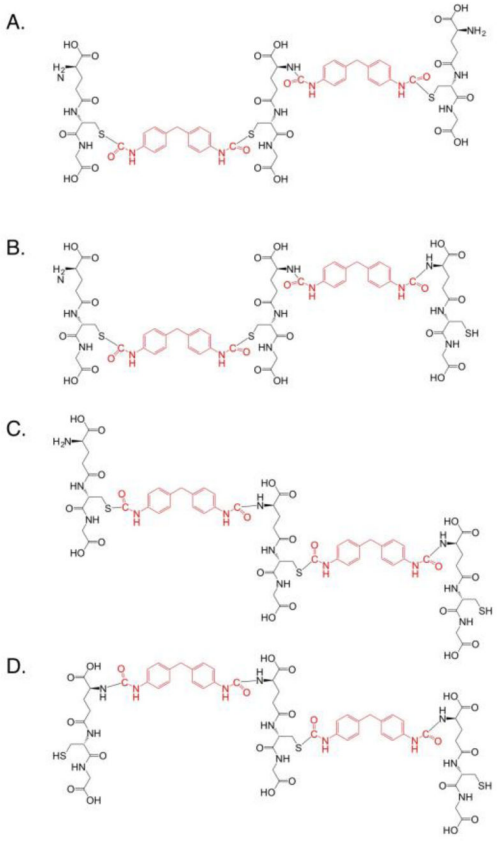

Figure 4.

Chemical predictions of possible GSH-MDI “oligomers”. Possible structures proposed for oligomeric GSH-MDI reaction products containing 3 GSH molecules (black) cross linked via N- or S-linkages by 2 MDI molecules (red) are depicted. LC-MS/MS data (see supplemental materials) support the configurations depicted in Panels A and B, but cannot rule out the presence of additional oligomers with configurations depicted in Panels C and D.

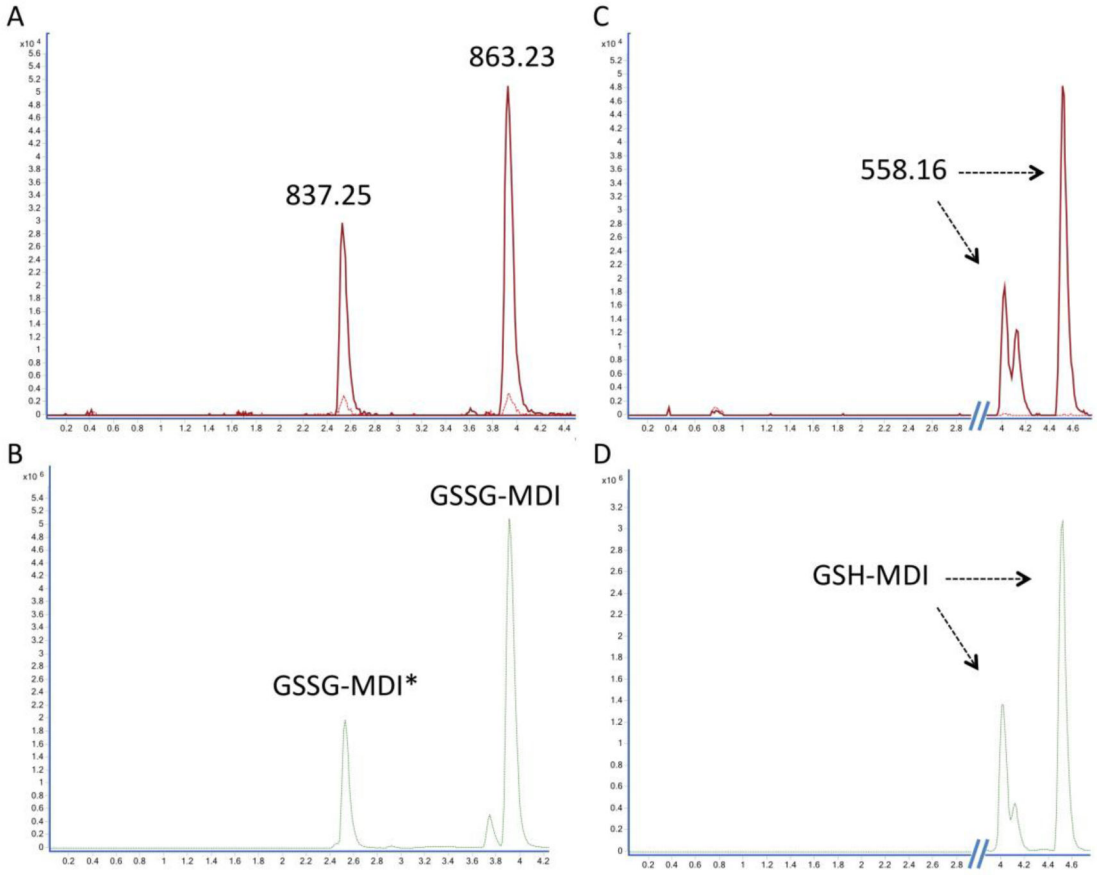

Figure 5.

Glutathione-MDI reaction products formed upon chemical incubation with liver S9 fractions. Extracted ion chromatograms (EICs) for $[M+H]^+$ ions with *m/z* ratios corresponding to GSSG-MDI and GSH-MDI are observed following incubation of MDI (solid lines), but not hydrolyzed MDI (dashed lines) with an S9 fraction (Panels A and C). Note identical elution profiles compared with GSSG-MDI and GSH-MDI reaction products formed with purified GSSG and GSH^i (B & D respectively). Data shown are representative of four independent experiments.